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THE MICROFUNGAL COMMUNITY OF TWO BLUEBERRY VARIETIES (*VACCINIUM MYRTHILLUS* L.) WITH *PESTALOTIOPSIS* SP. AS A DOMINANT TAXON

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Abstract: Two varieties of blueberry plants (i.e. Legacy and Bluegold) with fungal disease symptoms were evaluated to detect the phytopathogenic agent that caused the change of the regular morphology to "zebra stripes" and spots. Stems, leaves and shoots of four plant hosts were used as a source of biological material with a total of 114 plant fragments. The media used to isolate fungal microorganisms were yeast malt agar (YMA), corn meal agar (CMA), and organic substrate - carrots. 59 strains were isolated, grouped and identified as 11 organizational taxonomic units (OTUs), *Chaetomium* sp., *Fusarium* sp., *Alternaria* spp. and *Pestalotiopsis* sp. *Alternaria* strains were considered one organizational taxonomic unit (OTU) - *Alternaria* Section *alternata*. In plant organs, *Pestalotiopsis* recorded the highest colonization frequency in stems (CF% = 38.89) and shoots (CF% = 30.56). The highest colonization frequencies for *Pestalotiopsis* and *Alternaria* strains were obtained for the stem fragments on CMA - 75% and for the shoots on YMA - 66.67%, respectively. The frequency of colonization per plant host varies with values between 5% and 38.46% for *Pestalotiposis* sp., between 2.56% and 36.84% for *Alternaria* spp., and the rest of the taxa were represented by values between 2.5% and 10.53%. *Alternaria* colonized with a total frequency of 27.19% and *Pestalotiopsis* with a total frequency of 22.81%. The OTUs recorded low values (<4.5%), with a total colonization frequency of 20.18%. The registered values for fungal colonization rates per culture media were: 52.87% - carrot, 76.32% - CMA and 84.21% - YMA. Plant fragments varied in colonization rates between 57.7% in leaves and 83.33% in shoots. The colonization rate per total plants sample was 70.18%. The article describes morphological characters for the systematics of the *Pestalotiopsis* sp. strain, the potential phytopathogenic agent of blueberry.

Key words: *phytopathogens, shrubs, microorganisms*

INTRODUCTION

Lately, in Romania, blueberry (*Vaccinium myrthillus*) plantations gain interest among consumers to diversify their diet and to consume both fresh and processed fruits for their nutritional and antioxidant properties. Therefore, surfaces for cultivation have been extended.
For setting up blueberry crops, farmers face a major problem of procuring plant material from various countries, and this involves spreading of potential pathogens and their adaptation to the new environmental conditions. Several fungal pathogenic species of blueberry are considered cosmopolites like *Diaporthe* (Michalecka et al., 2017; Vilka & Volkova, 2015), *Botryosphaeria* (Elfar et al., 2013; Xu et al., 2015), *Neofusicoccum* (Espinoza et al., 2009). Species of *Pestalotiopsis* in association with dieback of blueberry were reported in Chile and Argentina (Espinoza et al., 2008), China (Zhao et al., 2014), Turkey (Dil et al., 2013; Erper & Celik, 2011), Mexico (Mondragón Flores et al., 2012; Rebollar-Alviter et al., 2013), Uruguay (Gonzalez et al., 2012), USA (Cline, 2014). *Pestalotiopsis* spp. are often isolated as endophytes (Liu et al., 2006; Watanabe et al., 2010; Wei et al., 2007), or occur as saprobes (Agarwal & Chauhan, 1988; Ho & Hyde, 2002; Hu et al., 2007; Liu et al., 2008; Wu et al., 1982).

Pathogenic *Pestalotiopsis* spp. was found on apple, blueberry, coconut, chestnut, ginger, grapevine, guava, hazelnut, lychee, mango, orchid, peach, rambutan, and tea (Maharachchikumbura et al., 2014).

The microfungal community living in blueberry plants with 2 varieties, Legacy and Bluegold, cultivated in Dambovita County was examined. Plants presented fungal disease symptoms: "zebra stripes" and leaf spots. The aim of the present paper is to determine the diversity of the microfungal community, to morphologically characterize *Pestalotiopsis* sp. and to evaluate the infection capacity of this taxon.

MATERIALS AND METHODS

Isolation of fungal strains. For the detection of potential pathogens, stems, leaves and shoots fragments were used. Red spots that evolved in reddish brown spots and lesions were observed on the leaves and in the necrotic areas. For the fungal isolation, 114 fragments of affected stems, shoots and leaves were used to isolate the potential pathogens and the neighbourhood fungal community. After sampling, the tissues were surface sterilized with distilled water, 70% ethanol, 15% sodium hypochlorite, 70% ethanol and sterile distilled water, one minute for each solution. Tissues were plated on solid media: yeast malt extract (YMA), corn meal (CMA) and carrots. Plates were incubated in the dark at 23 ± 0.5°C for a week and once fungal outgrowth was observed, purification of the strain was performed.

Fungal purification and maintenance. Purification of the fungal strains and further replication for morphological characterization were performed on various solid nutritive media: corn meal (CMA), potato dextrose (PDA), malt extract (MEA), organic substrate from 8 vegetables (CV8) and pine needles. Plates were incubated in the dark at 23 ± 0.5°C for 7 (±3) days (depending on the fungal growth speed). For the mycological collection (long-term conservation) only one isolate of the same taxon was maintained, in glycerol (20%) and mineral oil at -38°C and 5°C, respectively.

Calculation of diversity indices. The diversity of the microfungal community was calculated using two indices. The colonization rate (CR%) was calculated as the total number of stem fragments in a sample (i.e. plant, medium, plant fragment) yielding at least one isolate divided by the total number of stem fragments in that sample. CR = (number of colonized fragments/total number of fragments used) x 100. Colonization frequency (CF%) was calculated as the total number of fragments in a sample (plant, nutritive media, plant fragment) colonized by a species divided by the total number of fragments plated. CF% = (number of colonized species fragments or OTU/total number of fragments used) x 100.

Morphological characterization. Following the purification step, the fungal strains were analysed macro- and microscopically, and identified at genus level or as organizational taxonomic units (OTUs). Observations on the shape, colour and texture of the colonies were assessed for 14 strains tentatively identified as *Pestalotiopsis* spp. The shape, size and colour of conidiomata and conidia were analysed at microscope.

Observations on the fungal colony of *Pestalotipsis* sp. were performed on YMA, PDA and CMA. Carrot was the substrate for obtaining conidiomata and conidia used to observe the necessary characteristics for the systematics of *Pestalotiopsis*.

Fungal mycelium and spores were observed under the light microscope and photographed. All the microscopic measurements were determined with ToupView and 30 conidial measurements were taken (Maharachchikumbura et al., 2013). Measurements were made with minimum and maximum values and the confidence interval. The average and standard deviation were calculated (Maharachchikumbura et al., 2014).

Koch's Postulate. To detect the potential virulence as pathogenic agent of the *Pestalotiopsis* sp. strain, blueberry plants were infected. The infection was performed with a suspension of $1x10^5$ ml^{-1} conidia applied by spraying at a final volume of 2 ml/plant. The branches were covered with polyethylene bags for 72 hrs. A set of leaves was maintained as control using H_2O. The plants were cultivated in growth chamber for 15 days at a light - dark regime of 8:16 hrs (16-22°C; RH 80-90%). The light was maintained at 150 µmol x m^{-2} x sec^{-1}.

The test was evaluated isolating *Pestalotiopsis* sp. from the necrotic areas and calculating the attack rate. The assessments of the attack rate followed a visual disease severity index (Hafez et al., 2013).

Table 1. Disease severity index visualized on leaves

Characteristics	Interval for characteristics	Index
Without symptoms	-	
Very small spots	<10%	1
Small sports	11-25%	2
Large spots	26-50%	3

RESULTS AND DISCUSSIONS

The rate of colonization of various plant fragments was used to observe if the colonization differs among the organs. As it can be observed in the Figure 1, shoots were majorly colonized compared to leaves and stem with a difference of almost 25% between shoots and leaves. Also, differences were observed between nutritive media, with YMA as the most favourable medium for fungal outgrowth compared to carrot (approximately 34% more). Regardless of the plant organ and the nutritive medium used, the colonization rate per plant was 70%.

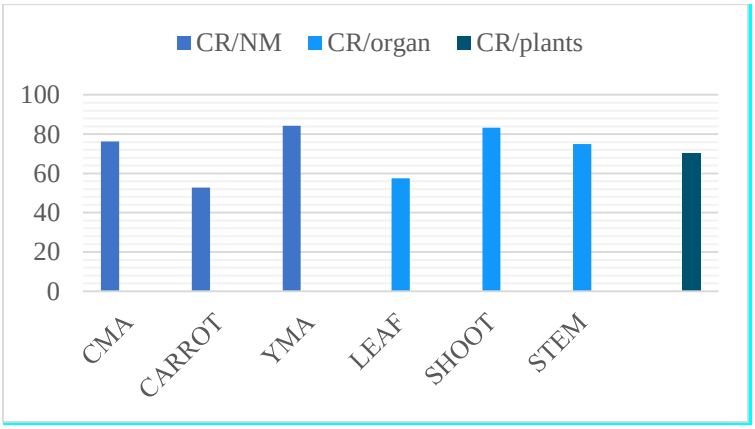

Figure 1. Colonization rate per nutritive medium, per plant organs and per total plants

After morphological observations and characterization, 59 strains were classified as 11 OTUs, *Fusarium* sp., *Chaetomium* sp., *Pestalotiopsis* sp. and *Alternaria* Section *alternata* (further *A. alternata*). In terms of colonization frequency per plants, no significant differences were observed between *Pestalotiopsis* sp. and *A. alternata* (Figure 2). It is noteworthy to mention that the sums of the values for colonization frequencies per plant, organ and medium did not reach 100% as uncolonized fragments were taken into account. Three fungal taxa were

isolated from symptomatic 1- to 2- year-old stems of blueberry in Chile: *Diaporthe* spp. (53%), *Botryosphaeriaceae* (38%) and *Pestalotiopsis* (9%) (Elfar et al., 2013).

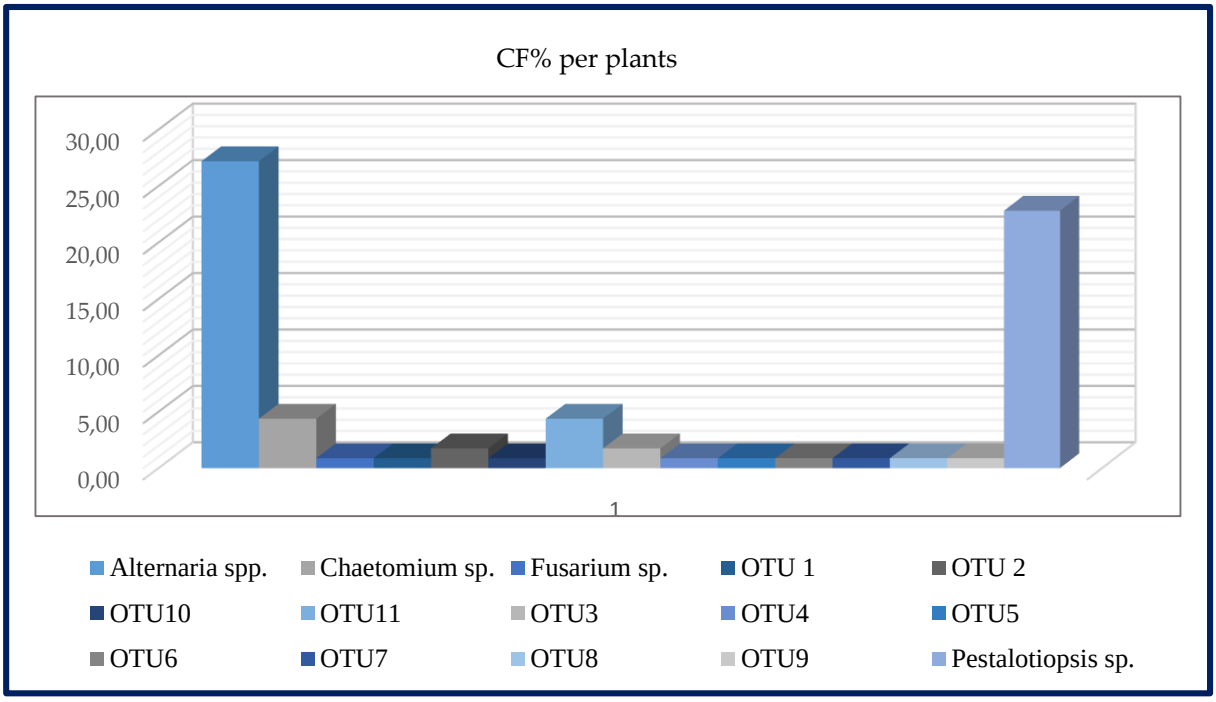

Figure 2. Colonization frequency per plants

The sums of the values for CF%, calculated for all taxa, with respect to CMA and YMA were 87% and 84%, respectively. No significant differences (Figure 3) were observed between *Pestalotiopsis* and *Alternaria* on CMA (CF = 29% versus CF = 24%, respectively). Yet, on YMA the frequency of *Alternaria* was slightly more than double than the value registered for *Pestalotiopsis* (CF = 47% versus CF = 21%). *Pestalotiopsis* mycelial growth was favoured on carrots – 19% of a total value of CF% = 39.

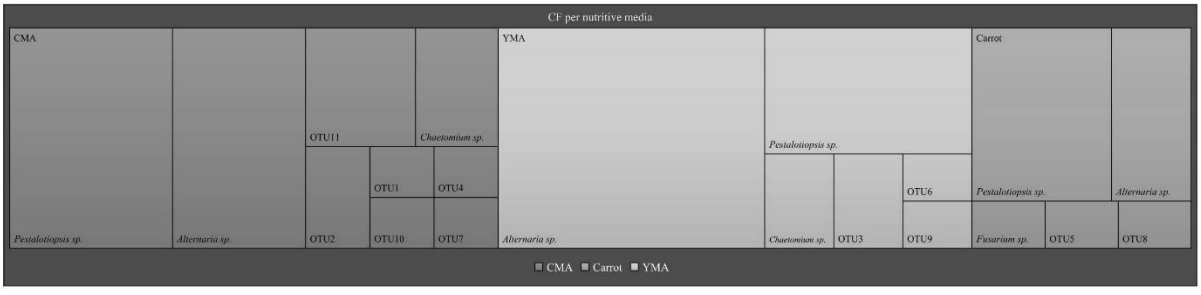

Figure 3. Colonization frequency per nutritive medium

In regards to plant organs, *Pestalotiopsis* recorded the highest colonization frequency in shoots (CF% = 30.56) and stems (CF% = 38.89) of total values of CF shoots = 50% and CF stems = 64%, calculated for all taxa. Leaves were less colonized by *Pestalotiopsis* sp. compared to *Alternaria* spp. and other OTUs (Figure 4). In a study on diseased blueberry plants, 50% of the fungal genera (*Alternaria*, *Colletotrichum*, *Neofusicoccum*, *Pestalotiopsis*) were found both in leaves and stems while *Curvularia*, *Phoma* and *Phomopsis* were isolated from stems and *Stemphylium* was found on leaves (Mondragón Flores et al., 2012).

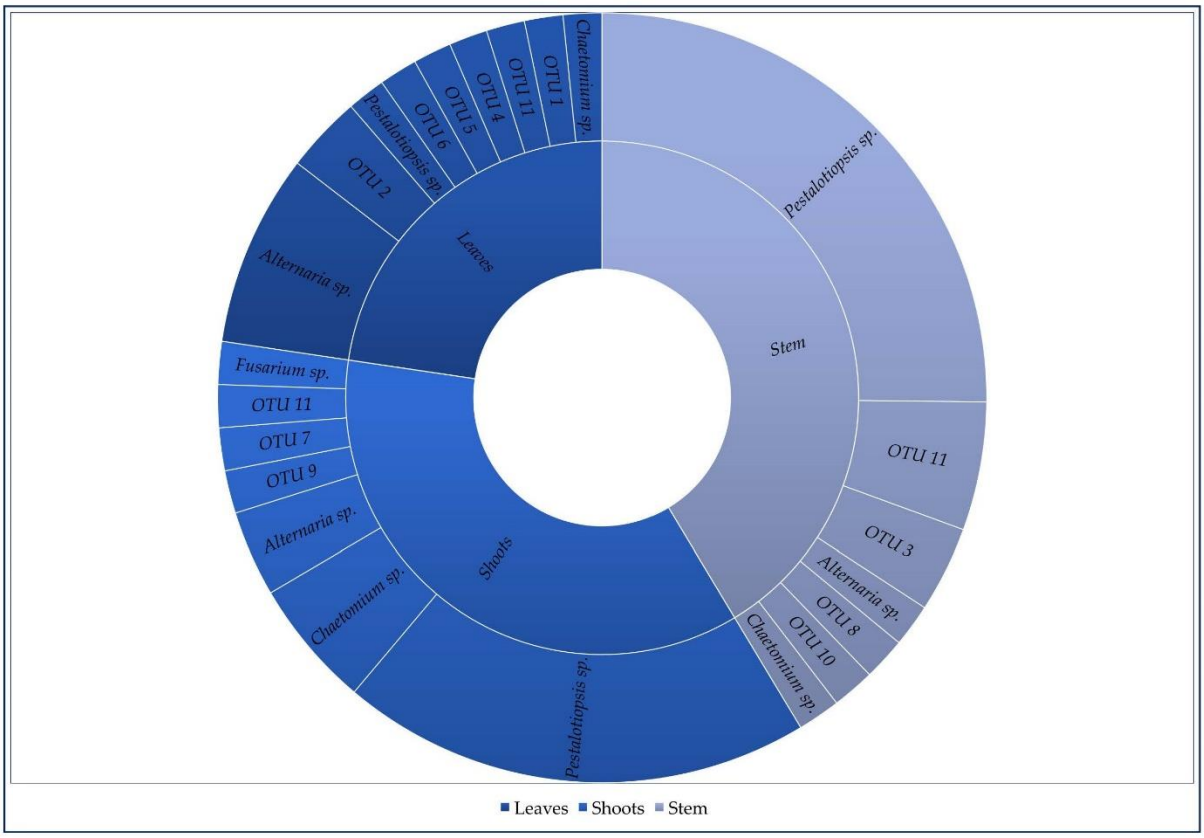

Figure 4. Colonization frequency per plant organs

The highest colonization frequencies for *Pestalotiopsis* and *Alternaria* strains were obtained for the stem fragments on CMA - 75% and for the shoots on YMA - 66.7%, respectively.

No nutritive media influenced positively the growth of fungi on leaves - all values ranged between 6.2% and 8.3%; except *Alternaria* on YMA (CF = 18.7%) and OTU 2 (CF = 14.3%). As it may be observed in the Figure 5, only *Pestalotiopsis* and *Alternaria* were isolated from multiple sets of combined nutritive media and plant organs; except *Chaetomium* sp. and OTU11, with three sets each other.

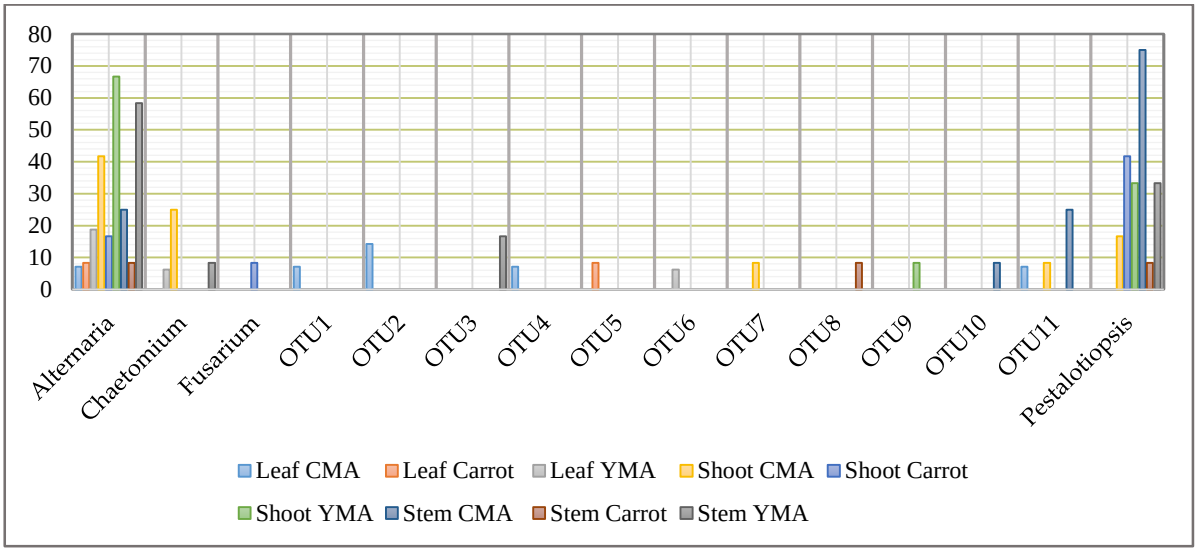

Figure 5. Colonization frequencies values (y axis) for all fungal taxa registered in combined nutritive medium and plant organ

Morphological characterization of *Pestalotiopsis* sp. isolates

Conidiomata in culture on carrot, globose to clavate, aggregated or dispersed on the medium surface, semi-immersed, dark brown, up to 700 µm diameter; exuding globose, dark brown to black conidial masses. Conidia straight to slightly curved, 4-septate, (18-)21-23(-27) × (5-)6-7(-10) µm, x ± SD = 22.3 ± 2 × 6 ± 1 µm; basal cell conic to obconic, hyaline and thin-walled; three median cells doliiform to subcylindrical, (13-)15-16(-17) µm long, x ± SD = 15 ± 1 µm, versicoloured, septa darker than the rest of the cell (second cell from the base pale brown, 3-9 µm long; third cell darker brown, 5-10 µm long; fourth cell pale brown to dark brown 3-5 µm long); apical cell 2-4 µm long, hyaline, subcylindrical to obconic and thin-walled; with 3 tubular apical appendages, arising from the apical crest, unbranched, filiform, flexuous, (13-)23-27(-31) µm long, x ± SD = 25 ± 5 µm; basal appendage single, tubular, unbranched, centric, 5-10 µm long.

Culture characteristics: colonies on PDA attaining 70 mm diameter after 7 d at 25°C, with crenate and undulated edge, whitish coloured, with dense aerial mycelium; reverse similar in colour. It is noteworthy to mention that when cultivated on pine needles (Figure 6), abundant conidiomata were developed (i.e. compared to all nutritive media).

Due to the colour intensity of the median cells, this strain can be included in the clade - versicolorous median conidial cells (Maharachchikumbura et al., 2012).

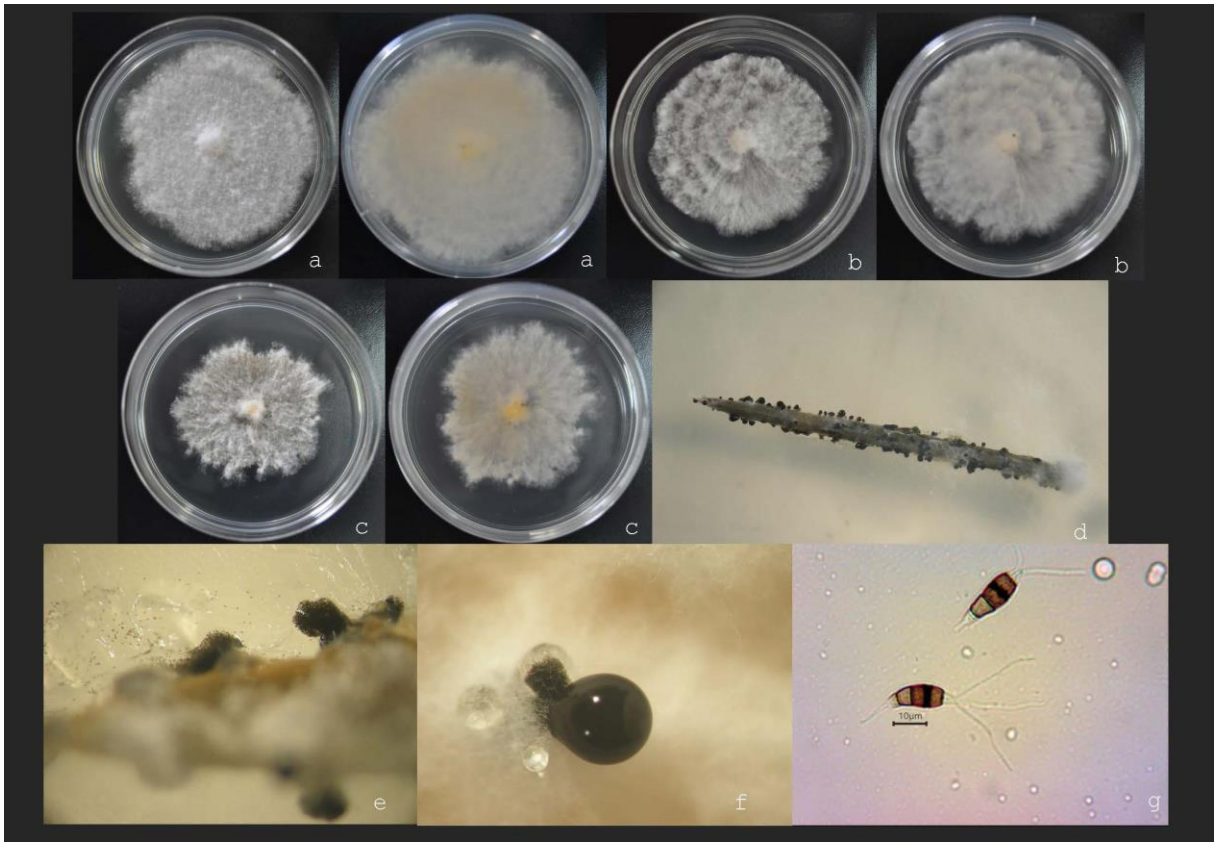

Figure 6. Fungal colonies of *Pestalotiopsis* on PDA (a), CMA (b) and YMA (c). Conidiomata on pine needles (d), conidia on pine needles (e), conidioma and conidia on carrot (f) and conidia at microscope (g)

Koch's Postulate

Assessments on 370 leaves were made to evaluate the pathogenic potential of a *Pestalotiopsis* sp. strain. The strain revealed a low-aggressive infection capacity under the tested conditions (80.3% of the leaves presented lack of symptoms). Almost two times less large spots were observed than very small spots (3.8% versus 7.8%). Differences between small and very small spots were not significant - 8.9% versus 7.8%, respectively. *Pestalotiopsis* spp. caused brown spots of 5.5-8 mm and 3-8 mm in diameter on wounded leaves after one month and 18 days, respectively, after inoculation (Dil et al., 2013). Browning of young shoot tips followed by blighting was observed on plants inoculated with *P. guepinii* (Erper & Celik, 2011).

CONCLUSIONS

The *Pestalotiopsis* strain presented morphological characteristics specific to the Clade - versicolorous median conidial cells, where eight species have been previously described. Identification of the species of *Pestalotiopsis* requires the use of molecular techniques. It is necessary to study blueberry plants from different geographical areas to analyze the relationship between potential pathogens (biotic) and abiotic conditions. Herein, *Pestalotiopsis* and *Alternaria* were observed as major colonizers of the plants. In the present evaluation the *Pestalotiopsis* taxon did not respond as a virulent pathogen. Further tests with changing abiotic and biotic conditions for the Koch's postulate may be required to obtain similar responses of the *Pestalotiopsis* strain.

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